International Publication No. WO 93/15200 A1

Job No.: 1074-85207 Ref.: 06832.0004-02000
Translated from French by the Ralph McElroy Translation Co.
910 West Avenue, Austin, Texas, 78701

INTERNATIONAL PATENT OFFICE
WORLD ORGANIZATION FOR INTELLECTUAL PROPERTY
International patent published on
the basis of the Patent Cooperation Treaty
INTERNATIONAL PUBLICATION NO. WO 93/15200 A1[1]

International Patent Classification[5]:	C 12 N	15/12
		15/62
		15/14
	C 12 N	5/10
	A 61 K	37/02
International Filing No.:	PCT/FR93/00087	
International Filing Date:	January 28, 1993	
International Publication Date:	August 5, 1993	
Priority		
Date:	January 31, 1992	
Country:	FR	
No.:	92/01066	
Designated States:	CA, FI, JP, NO, US, European Patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE)	

ANTITHROMBOTIC POLYPEPTIDES AS ANTAGONISTS OF THE BINDING OF vWF TO PLATELETS OR TO SUBENDOTHELIUM

[1] [p. 29 of the patent document and pp. 1-4,7-11 of the figures are replacement sheets]

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Published
With International Search Report.

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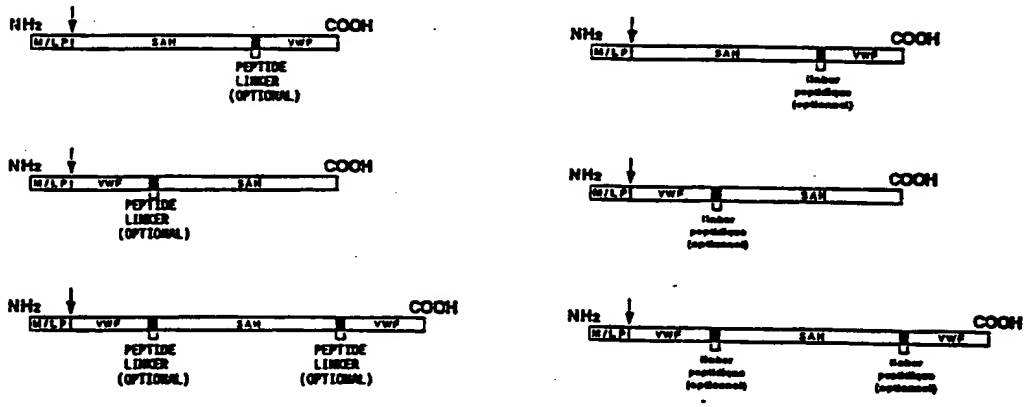

(57) Abstract

Recombinant polypeptides consisting of an adhesive portion derived from the structure of the vWF which is at least partially antagonistic to the bond between said vWF and the platelets and/or the subendothelium, as well as a portion for stabilising and presenting it *in vivo*; preparation thereof; and pharmaceutical compositions containing said polypeptides.

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The present invention concerns novel antithrombotic polypeptides, their preparation and pharmaceutical compositions containing them. More specifically, the present invention concerns novel polypeptides comprising a part derived from the structure of the von Willebrand's factor (vWF) which is intrinsically capable of binding to blood platelets and/or the subendothelium.

vWF is a glycosylated protein comprising 2813 amino acids which contains a 22 residue signal sequence, a 741 residue "pro" region and a 2050 amino acid mature protein organized into several repeated structures (Titani, K. et al., Biochemistry 25 (1986) 3171-3184; Verweij, C. L. et al., EMBO J. 5 (1986), 1839-1847). This complex glycoprotein is present in vivo, or it is stored in specialized vesicles of the endothelial cells or of the platelets, or in a circulating form in the blood plasma after secretion and proteolytic maturation during the secretion process. The circulating forms of vWF are present in the form of high molecular weight multimers (up to 20,000 kd), and whose protomer is a dimer of approximately 450 kd. The vWF gene was cloned and sequenced by several teams and mapped on the short arm of the chromosome 12 (Sadler, J. E. et al., Proc. Natl. Acad. Sci. 82 (1985) 6394-6398; Verweij, C. L. et al., EMBO J. 5 (1986) 1839-1847; Shelton-Inloes B.B. et al., Biochemistry 25 (1986) 3164-3171; Bonthron D. et al., Nucleic Acids Res. 17 (1986) 7125-7127; Ginsburg, D. et al., Science 228 (1985) 1401-1406).

vWF is involved in the genesis of arterial thrombi by a complex interaction which is poorly understood between certain components of the subendothelium on the one hand and the blood platelets on the other hand (and notably the platelet GP1b receptors). An important point is that circulating plasma vWF does not spontaneously bind to the GP1b receptors of the platelets, and it is likely that its interaction with the subendothelium is necessary to unmask its interaction site(s) with the platelets, for example, after a conformational change of the vWF. The interaction between the vWF these activated and the platelet GP1b leads to the activation of the blood platelets, which then acquire the capacity to aggregate and to generate a fibrinocellular thrombus in the presence of certain adhesive proteins (fibrinogen, thrombospondin, vWF, etc.).

Taking into account its early role in platelet activation, vWF constitutes a pharmacological target of choice for the preparation of antithrombotic agents. However, numerous difficulties have to be overcome to be able to exploit this molecule on the pharmacological level: the incapacity of the circulating vWF to bind the platelets, the lack of knowledge on the respective contribution of the different adhesive functions of vWF (subendothelium and platelets) in its thrombogenic activity, the difficulty of producing sufficiently large amounts of sufficiently pure and homogeneous products for use as therapeutic agents, the large size of vWF and its complexity, the dynamics of its tertiary structure, etc. Some fragments of vWF have been obtained by proteolytic digestion and studied on the pharmacological level. Recombinant fragments have also been produced (EP 255 206; Sugimoto, M. et al., Biochemistry 30 (1991) 5202-5209; Azuma, H. et al., J. Biol. Chem. 266 (1991)

12342-12347). It is apparent from these studies that the molecules obtained are not entirely satisfactory, and in particular that they do not behave as optimal antagonists of the vWF-platelet interaction in the absence of certain nonphysiological ligands (such as, for example, ristocentin or botrocetin), or they have to be chemically modified (for example, reduction and alkylation), probably to unmask the cryptic binding sites of vWF to platelet GP1b.

The present invention provides novel molecules which are intrinsically capable of at least partially antagonizing platelet activation. The molecules of the invention comprise an adhesive part which is derived from the structure of vWF and a part which allows its functional presentation and ensures the stability and the distribution of the molecule in vivo. Indeed, the applicant has shown that it is possible to genetically couple vWF with a structure of the protein type and to produce such molecules at satisfactory levels. In addition, the molecules of the invention allow the generation and the use of small structures derived from vWF, and which are thus very specific for a desired effect (for example, antagonists of the vWF-GP1b interaction alone). Moreover, the applicant has shown that such a coupling promotes the presentation of this structure at its binding site(s). The polypeptides of the invention thus make it possible to expose, within a stable structure, structures derived from vWF which are capable of at least partially antagonizing the bond of vWF to the platelets, and as a result, of inhibiting platelet activation. The polypeptides of the invention also make it possible to expose, within a stable structure, structures derived from vWF which are capable of at least partially antagonizing the bond of vWF to the subendothelium.

A subject of the present invention therefore concerns molecules comprising an adhesive part derived from the structure of vWF, capable of at least partially antagonizing the binding of vWF to the platelets and/or the subendothelium, and a part of a protein nature allowing its in vivo stabilization and presentation.

More specifically, in the molecules of the invention the adhesive part consists in its entirety or in part of the peptide sequence between the residues 445-733 of vWF or one of its variants. The peptide sequence of vWF having been published, the numbering of the residues of the adhesive part of the molecules of the invention refers to the numbering of the sequence of vWF published by Titani et al. (Biochemistry 25 (1986) 3171-3184). It is understood that this function can be redundant within molecules of the present invention. A part of this sequence of vWF (residues Thr470 to Val713) is indicated in Figure 1, in which human serum albumin is coupled at the C-terminal.

In the meaning of the present invention, the term "variant" denotes any molecule obtained by the modification of the sequence which is capable of at least partially antagonizing the binding of vWF to the platelets and/or the subendothelium. The expression "modification" denotes any mutation, substitution, deletion, addition or modification obtained, for example, by

the techniques of genetic engineering. Such variants can be generated for different purposes, such as, notably, to increase the affinity of the molecule for its binding site(s), to improve its production levels, to reduce its susceptibility to proteases, to increase its therapeutic efficacy or to reduce its side effects, or to confer novel pharmacokinetic or biological properties to it, notably adhesive functions which are intrinsically expressed in a noncryptic manner.

Particularly advantageous polypeptides of the invention are those in which the adhesive part presents:

(a) the peptide sequence between the residues 445-733 of vWF, or

(b) a part of the peptide sequence (a), capable of at least partially antagonizing the binding of vWF to GPb1 and/or the subendothelium, or

(c) a structure derived from structures (a) or (b) by structural modifications (mutation, substitution, addition and/or deletion of one or more residues) and capable of at least partially antagonizing the binding of vWF to GPb1 and/or the subendothelium, or

(d) a non-natural peptide sequence, for example, one isolated from peptide banks and capable of at least partially antagonizing the binding of vWF to GP1b and/or the subendothelium.

Among the structures of type (b) one can mention more specifically those that preserved the capacity of antagonizing the interaction between vWF and platelet GP1b, such as, for example, the peptides G10 or D5 described by Mori et al. (J. Biol. Chem. 263 (1988) 17901-17904), or the peptides which preserved their capacity to bind to collagen (Pareti, F. I. et al., J. Biol. Chem. 261 (1986) 15310-15315; Roth, G. J. et al., Biochemistry 25 (1986) 8357-8361), and/or heparin (Fujimura, Y. et al., J. Biol. Chem. 262 (1987) 1734-1739), and/or botrocetin (Sugimoto, M. et al., J. Biol. Chem. 266 (1991) 18172-18178), and/or the sulfatides (Christophe, O. et al., Blood 78 (1991) 2310-2317), and/or ristocentin, etc..., or any combination between these different adhesive structures.

The structures of type (c) comprise, for example, the molecules in which certain N- or O-glycosylation sites were modified or suppressed, as well as the molecules in which one or more or all the cysteine residues were substituted, or point and/or multiple mutants concerning at least one residue involved in pathologies of type IIB associated with the vWF, such as the Arg543, Arg545, Trp550, Val553 or Arg578 residues, for example. They also comprise molecules obtained from (a) or (b) by deletion of regions that do not intervene or intervene little in the interaction with the binding sites considered, and molecules comprising, compared to (a) or (b), additional residues such as, for example, an N-terminal methionine and/or a secretion signal sequence and/or a polypeptide adapter allowing the joining to the stabilizing structure.

As an example, one can cite polypeptides of the invention comprising the stabilizing structure coupled:

- to a peptide of type P1, whose minimum version corresponds to the peptide G10 between the Cys474 and Pro488 residues of vWF, or

- to a peptide of type P2 whose minimal version corresponds to the peptide D5 between the Leu694 and Pro708 residues of vWF, or

- to a peptide of type X or XD corresponding, respectively, to the fragment of vWF between the Pro488 and Leu694 residues, and its variants obtained by deletion, or

- to a peptide of type X* defined as any molecular variant of the peptides of type X and XD, or

to any combination of these peptides, and, among others:

- the peptides of type P1-P2;

- the peptides of type P1-X, P1-XD, P1-X*;

- the peptides of type X-P2, XD-P2, X*-P2;

- the peptides of type P1-X-P2;

- the peptides of type P1-XD-P2;

- the peptides of type P1-X*-P2;

- any adhesive peptide as defined above and represented more than once within the molecule of the invention.

The adhesive part of the molecules of the invention can be coupled, directly, or through the intermediary of a joining peptide to the stabilizing protein structure. In addition, it can form the N-terminal end or the C-terminal end of the molecule. It is preferred in the molecules of the invention for the adhesive part to constitute the C-terminal part of the chimera.

It is preferred for the stabilizing structure of the polypeptides of the invention to be a polypeptide having a high plasma half-life. For example, it can be a protein such as an albumin, an apolipoprotein, an immunoglobin or a transferrin, etc... It can also consist of peptides derived from such proteins by structural modifications, or artificially or semiartificially synthesized peptides having a high plasma half-life. Moreover, the stabilizing structure used is given preference over a weakly immunogenic or nonimmunogenic polypeptide for the organism in which the polypeptide of the invention is used.

In a particularly advantageous embodiment of the invention, the stabilizing structure is an albumin or a variant of albumin, for example human serum albumin (HSA). It is understood that the variations of the albumin denote any protein with high plasma half-life obtained by modification (mutation, deletion and/or addition) by the techniques of genetic engineering of a gene coding for a given isomorph of human serum albumin, as well as any macromolecule with high plasma half-life obtained by in vitro modification of the protein coded for by such genes. Since albumin is very polymorphous, numerous natural variants have been identified and listed (Weitkamp, L. R. et al., Ann. Hum. Genet. 37 (1973) 219). For example, the chimeras between

said adhesive function(s) and mature HSA possess pharmaceutical properties and antithrombotic activities which are particularly useful in therapy.

Another subject of the invention concerns a method for the preparation of the above-described chimeric molecules. More specifically, this method consists in having a eukaryotic or prokaryotic cell host express a nucleotide sequence coding for the desired polypeptide, and then in harvesting the polypeptide produced.

Among the eukaryotic hosts that can be used in the context of the present invention, one can mention animal cells, yeasts or fungi. In particular, in the case of yeasts, one can mention the yeasts of the genera *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Schwanniomyces*, or *Hansenula*. In the case of animal cells, one can mention the COS, CHO, C127 cells, etc... Among the fungi that can be used in the present invention, one can cite, more specifically, *Aspergillus* ssp. [sic; spp.] or *Trichoderma* ssp. As prokaryotic hosts, it is preferred to use the bacteria such as *Escherichia coli*, or those belonging to the genera *Corynebacterium*, *Bacillus* or *Streptomyces*.

The nucleotide sequences that can be used in the context of the present invention can be prepared in different manners. In general, they are obtained by assembling in a reading frame the sequences which code for each one of the functional parts of the polypeptide. The latter can be isolated by the techniques known to a person skilled in the art, for example directly from cellular messenger RNA (mRNA), or by recloning from a complementary DNA bank (cDNA) prepared from producing cells, or they can be completely synthetic nucleotide sequences. Moreover, it is understood that the nucleotide sequences can also be modified later, for example by the techniques of genetic engineering, in order to obtain derivatives or variants of said sequences.

More advantageously, in the process of the invention, the nucleotide sequence is a part of an expression cassette comprising a transcription initiation region (promoter region) which allows, in the host cells, the expression of the nucleotide sequence placed under its control and coding for the polypeptides of the invention. This region can originate from promoter regions of genes that are strongly expressed in the host cell used, the expression being constitutive or regulable. In the case of yeasts, the promoter can be the promoter of phosphoglycerate kinase (PGK), of glyceraldehyde-3-phosphate dehydrogenase (GPD) gene, of lactase (LAC4), of the enolases (ENO), of the alcohol dehydrogenases (ADH) genes, etc... In the case of bacteria, the promoter can be the right or left promoter of the genes of bacteriophage lambda (P_L, P_R), or promoters of the genes of the tryptophan (P_{trp}) or lactose (P_{lac}) operons. In addition, this control region can be modified, for example by in vitro mutagenesis, by the introduction of additional control elements or synthetic sequences, or by deletions or substitutions of the original control elements. The expression cassette can also comprise a region of termination of the functional transcription in the host considered, positioned immediately downstream of the nucleotide sequence coding for a polypeptide of the invention.

In a preferred method, the polypeptides of the invention result from the expression of a eukaryotic or prokaryotic host of a nucleotide sequence and the secretion of the expression product of said sequence in the culture medium. Indeed, it is particularly advantageous to be able to obtain, by a recombinant route, the molecules directly in the culture medium. In this case, the nucleotide sequence coding for a polypeptide of the invention is preceded by a "leader" sequence (or signal sequence) directing the nascent polypeptide into the secretory routes of the host used. This "leader" sequence can be the natural signal sequence of vWF or the stabilizing structure in the case where it is a naturally secreted protein, but it can also be any other functional "leader" sequence, or an artificial "leader" sequence. The choice of either one of these sequences is notably dependent on the host used. Examples of functional signal sequences include those of the genes of sexual pheromones or of the "killer" toxins of yeasts.

In addition to the expression cassette, one or more markers which allow the selection of the recombinant host can be added, such as for example the URA3 gene of *S. cerevisiae*, or genes which confer resistance to antibiotics such as geneticin (G418), or any other toxic compound, such as certain metal ions.

The assembly constituted the expression cassette and the selection marker can be introduced either directly into the host cells considered, or they can be first inserted into a functional self-replicating vector. In the first case, the sequences which are homologous to regions present in the genome of the host cells are preferably added to this assembly; said sequences being then positioned on each side of the expression cassette and of the selection gene so as to increase the frequency of integration of the assembly into the genome of the host by targeting the integration of the sequences by homologous recombination. In the case where the expression cassette is inserted in a replicating system, a preferred replication system for yeasts of the genus *Kluyveromyces* is derived from the plasmid pKD1, which was initially isolated from *K. drosophilarum*; a preferred replication system for yeasts of the genus *Saccharomyces* is derived from the plasmid 2μ of *S. cerevisiae*. In addition, this expression plasmid can contain all or part of said replication systems, or it can combine elements derived from the pKD1 plasmid as well as from the plasmid 2μ.

In addition, the expression plasmids can be shuttle vectors between a bacterial host such as *Escherichia coli* and the chosen host cell. In this case, a replication origin and a selection marker which function in the bacterial host are required. It is also possible to position restriction sites surrounding the bacterial and unique sequences on the expression vector: this allows the elimination of these sequences by cutting and religation in vitro of the truncated vector before transformation of the host cells, which can result in an increase in the number of copies and in an increased stability of the expression plasmids in said hosts. For example, such restriction sites can correspond to sequences such as 5'-GGCCNNNNNGGCC-3" (SfiI) or 5'-GCGGCCGC-3'

(NotI) to the extent that the sites are extremely rare and generally absent from an expression vector.

After the construction of such vectors or expression cassettes, the latter are introduced into the chosen host cells using classic techniques described in the literature. In this regard, any method which allows the introduction of a foreign DNA into a cell can be used. They can notably be transformation, electroporation, conjugation, or any other technique known to a person skilled in the art. As an example of hosts of the yeast type, the different strains of *Kluyveromyces* used were transformed by treating the whole cells in the presence of lithium acetate and polyethylene glycol according to the technique described by Ito et al. (J. Bacteriol. 153 (1983) 163). The transformation technique described by Durrens et al. (Curr. Genet. 18 (1990) 7), using glycol and dimethyl sulfoxide was also used. It is also possible to transform the yeasts by electroporation according to the method described by Karube et al. (FEBS Letters 182 (1985) 90). An alternative protocol is also described in detail in the following examples.

After the selection of the transformed cells, the cells expressing said polypeptides are inoculated, and the recovery of said polypeptides can be done either during cell growth by "continuous" processes, or at the end of growth for "batch" cultures. The polypeptides which are the subject of the present invention are then purified from the culture supernatant for their molecular, pharmacokinetic and antithrombotic characterization.

A preferred expression system of the polypeptides of the invention consists of the use of yeasts of the genus *Kluyveromyces* as the host cell, transformed by certain vectors derived from the extrachromosomal replicon pKD1 initially isolated in *K. marxianus* var. *drosophilarum*. These yeasts, particularly *K. lactis* and *K. fragilis*, are generally capable of replicating said vectors in a stable manner and in addition they have the advantage of being included in the list of G.R.A.S. ("Generally Recognized As Safe") organisms. Yeasts are preferentially industrial strains of the genus *Kluyveromyces* capable of replicating in a stable manner said plasmids derived from the plasmid pKD1, and in which a selection marker was inserted, as well as an expression cassette allowing the secretion of the polypeptides of the invention at high levels.

The present invention also concerns the nucleotide sequences coding for the above-described chimeric polypeptides, as well as the eukaryotic or prokaryotic recombinant cells comprising such sequences.

The present invention also concerns the application as a drug of the polypeptides according to the present invention. More specifically, the subject of the invention is any pharmaceutical composition comprising one or more polypeptides as described above. More specifically, these compositions can be used for the prevention or the treatment of thromboses.

The present invention will be described more completely with the aid of the following examples, which must be considered as illustrative and nonlimiting.

List of the figures

The representations of the plasmids indicated in the following figures are not drawn to scale, and only the restriction sites which are important for understanding the cloning operations carried out are indicated.

Figure 1: Nucleotide sequence of a restriction fragment HindIII coding for a chimeric protein of the type HSA-vWF. The black arrows indicate the end of the "pre" and "pro" regions of the HSA. The MstII and PstI restriction sites are underlined. The numbering of the amino acids (right column) corresponds to the mature chimeric protein HSA-vWF470->713 (829 residues); the fragment Thr470-Val713 of vWF of this particular chimera is numbered from the Thr586 to Val829 residue. The Thr470, Leu494, Asp498, Pro501, Tyr508, Leu694, Pro704 and Pro708 residues of mature vWF are underlined.

Figure 2: Schematic representation of chimeras of the type HSA-vWF (A), of the type vWF-HSA (B) or vWF-HSA-vWF (C). Abbreviations used: M/LP, translation initiator methionine residue, optionally followed by a secretion signal sequence; HSA, mature human serum albumin or one of its variants; vWF, a fragment(s) of vWF possessing the property of binding to platelets and/or the subendothelium, or a variant obtained by the techniques of genetic engineering. The black arrow indicates the N-terminal end of the mature protein.

Figure 3: A, restriction map of the plasmid pYG105 and strategy for the construction of the expression plasmids of the chimeric proteins of the present invention. Abbreviations used: P, transcription promoters; T, transcription terminator; IR, inverted repeated sequences of the plasmid pKD1; LP_{HSA}, "prepro" region of HSA; Ap^r and Km^r denote the genes of resistance to ampicillin (*E. coli*) and G418 (yeasts), respectively.

B, characteristics and genetic relationship of the principal expression plasmids of the hybrids between HSA and vWF exemplified in the present invention. The plasmids of the first column are plasmids of the pUC type comprising a HindIII restriction fragment corresponding to translational fusions between the totality of the HSA and a fragment or a molecular variant of vWF. The expression plasmids correspond to cloning in the productive orientation of these HindIII fragments in the HindIII site of the plasmid pYG105 (LAC4).

Figure 4: Characterization of the material secreted after 4 days of culture (Erlenmeyer flasks) of the strain CBS 293.91 transformed by the plasmids pYG1248 (expression plasmid of a chimera of the type HSA-P1-X-P2) and pKan707 (control plasmid). In this experiment, the results of panels A, B and C had migrated on the same gel (8.5% SDS-PAGE), and then treated separately.

A, staining with Coomassie blue; molecular weight standard (lane 2); supernatant equivalent to 50 µL of the culture transformed by the plasmids pKan707 in YPL medium (lane 1), or pYG1248 in YPD medium (lane 3) or YPL (lane 4).

B, immunological characterization of the material secreted after the use of mouse antibodies directed against human vWF: same legend as for A, except that biotinylated molecular weight standards were used.

C, immunological characterization of the material secreted after the use of rabbit antibodies directed against human albumin; supernatant equivalent to 50 µL of the culture transformed by the plasmids pKan707 in YPL medium (lane 1), or pYG1248 in YPD medium (lane 2) or YPL (lane 3).

Figure 5: Kinetics of secretion of a chimera of the type HSA-P2 by the strain CBS 293.91 transformed by the plasmid pYG1206.

A, staining with Coomassie blue; molecular weight standard (lane 1); supernatant equivalent to 2.5 µL of a "Fed Batch" culture in YPD medium after 24 h (lane 2), 40 h (lane 3) or 46 h (lane 4) of growth.

B, immunological characterization of the material secreted after use of mice antibody directed against human vWF: same legend as for A except that biotinylated molecular weight standards were used.

Figure 6: Characterization of the material secreted by *K. lactis* transformed by the plasmids pKan707 (control plasmid, lane 2), yPG1206 (expression plasmid of a chimera of the type HSA-P2, lane 3), pYG1206 (expression plasmid of a chimera of type HSA-P2, lane 3), pYG1214 (expression plasmid of a chimera of the type HSA-P1, lane 4) and PYG1223 (expression plasmid of a chimera of the type HSA-P1-XD-P2, lane 5); molecular weight standard (lane 1). The deposits correspond to 50 µL of supernatant of a stationary culture after growth in YPD medium, migration in an 8.5% acrylamide gel and staining with Coomassie blue.

Figure 7: Characterization of the material secreted after 4 days of culture (Erlenmeyer flasks) of the strain CBS 293.91 transformed by the plasmids pYG1311 (HSA-vWF508->704) and pYG1313 (HSA-vWF470->704, C471G, C474G), after migration on an 8,5% SDS-PAGE gel.

A, staining with Coomassie blue; supernatant equivalent to 100 µL of the culture transformed by the plasmids pYG1311 (lane 1) or pYG1313 (lane 2) in YPL medium; molecular weight standard (lane 3).

B, immunological characterization of the material secreted after the use of mice antibodies directed against human vWF: same legend as for A.

C, immunological characterization of the material secreted after the use of rabbit antibodies directed against human vWF; same legend as for A.

Figure 8: Characterization of the material secreted after 4 days of culture (Erlenmeyer flasks) of the strain CBS 293.91 transformed by the plasmids pYG1361 (HSA-vWF470->704, C471G, C474G, R543W) and pYG1365 (HSA-vWF470-<704, C471G, C474G, P574L), after migration on an 8.5% SDS-PAGE gel. In this experiment, the results of panels A, B and C had migrated in the same gel, then treated separately.

A, staining with Coomassie blue; supernatant equivalent to 100 µL of the culture transformed by the plasmids pYG1361 (lane 1) or pYG1365 (lane 2) in YPL medium; molecular weight standard (lane 3).

B, immunological characterization of the material secreted after use of mouse antibodies directed against human vWF: same legend as for A.

C, immunological characterization of the material secreted after use of rabbit antibodies directed against ASA; same legend as for A.

Figure 9: In vitro assay of the antagonistic activity for agglutination of human platelets fixed by paraformaldehyde: IC_{50} of the hybrids HSA-vWF694-708 (HSA-vWF470-713 C471G, C4746) and (HSA-vWF470-704 C471G, C474G) with respect to the standard RG12986. The determination of the dose-dependent inhibition of the platelet agglutination is carried out under stirring at 37°C using a PAP-4 aggregometer, in the presence of human vWF, of botrocetin (8.2 mg/mL) and of the product to be tested at different dilutions. The concentration of the product which allows the inhibition of half of the control agglutination (absence of product) is then determined (IC_{50})

Examples

General cloning techniques

The methods which are classically used in molecular biology, such as the preparative extractions of plasmid DNA, the centrifugation of plasmid DNA in a cesium chloride gradient, the electrophoresis on agarose or acrylamide gels, the purification of DNA fragments by electroelution, the extractions of proteins with phenol or phenol-chloroform, the precipitation of DNA in a saline medium by ethanol or isopropanol, the transformation in *Escherichia coli*, etc., are well known to a person skilled in the art and they have been extensively described in the literature (Maniatis, T. et al., "Molecular Cloning, a Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982; Ausubel, F. M. et al. (eds), "Current Protocols in Molecular Biology," John Wiley & Sons, New York, 1987).

The restriction enzymes were supplied by New England Biolabs (Biolabs), Bethesda Research Laboratories (BRL) or Amersham, and they are used according to the recommendations of the suppliers.

The plasmids of the type pBR322, pUC and the phages of the M13 series are of commercial origin (Bethesda Research Laboratories).

For the ligations, the DNA fragments are separated by size by electrophoresis on agarose or acrylamide gels, extracted with phenol or with a phenol/chloroform mixture, precipitated with ethanol, and then incubated in the presence of the phage T4 DNA ligase (Biolabs) according to the recommendations of the supplier.

The filling of the projecting 5' ends is carried out by the Klenow fragment of DNA polymerase I of *E. coli* (Biolabs) according to the specifications of the supplier. The destruction of the projecting 3'-ends is carried out in the presence of the phage T4 DNA polymerase (Biolabs), used according to the recommendations of the manufacturer. The destruction of the projecting 5'-ends is carried out by a controlled treatment with S1 nuclease.

The in vitro mutagenesis directed by synthetic oligodeoxynucleotides is carried out according to the method developed by Taylor et al. (Nucleic Acids Res. 13 (1985) 8749-8764), using the kit distributed by Amersham.

The enzymatic amplification of DNA fragments using the PCR technique (Polymerase-catalyzed Chain Reaction, Saiki, R. K. et al., Science 230 (1985) 1350-1354; Mullis, K. B. and Faloona, F. A., Meth. Enzym. 155 (1987) 335-350) is carried out using a DNA thermal cycler (Perkin Elmer Cetus) according to the specifications of the manufacturer.

The verification of the nucleotide sequences is carried out by the method developed by Sanger et al. (Proc. Natl. Acad. Sci. USA, 74 (1977) 5463-5467) using the kit distributed by Amersham.

The numbering of the amino acids of vWF is that of Titani et al. (Biochemistry 25 (1986) 3171-3184).

The transformations of *K. lactis* with the DNA of the expression plasmids of proteins of the present invention are carried out by any technique known to a person skilled in the art, an example of which is given in the text.

Unless otherwise indicated, the bacterial strains used are *E. coli* MC1060 (lacIPOZYA, X74, galU, galK, $strA^r$) or *E. coli* TG1 (lac, proA,B, supE, thi, hsdD5/FtraD36, $proA^+B^+$, $lacI^q$, lacZ, M15).

The yeast strains used belong to the budding yeasts, and more specifically to the yeasts of the genus *Kluyveromyces*. The strain *K. lactis* MW98-8C (a, uraA, arg, lys, K^+, pKD1°) and *K. lactis* CBS 239.91 were used in particular; a sample of strain MW98-8C was deposited on September 16, 1988 at Central Bureau For Fungal Cultures (CBS) at Baarn (Netherlands) where it is registered under number CBS 579.88.

A bacterial strain (*E. coli*) transformed by the plasmid pET-8c52K was deposited on April 17, 1990 at the American Type Culture Collection under the number ATCC 68306.

The yeast strains transformed by the expression plasmids coding for the proteins of the present invention are cultured in Erlenmeyer flasks or in 2 L pilot fermenters (SETRIC, France) at 28°C in a rich medium (YPD: 1% yeast extract, 2% Bactopeptone, 2% glucose; or YPL: 1% yeast extract, 2% Bactopeptone, 2% lactose) under constant stirring.

Example 1: Construction of the plasmid pET-8c52K and of its molecular variants

The cDNA fragment of vWF coding for residues 445-733 of human vWF has several determinants which are crucial for the interaction between vWF and the platelets on the one hand and certain elements of the basal membrane and of the subendothelium tissue on the other hand. The amplification of these genetic determinants can be carried out, for example, from a human cell line expressing vWF, and, for example, from a line of endothelial cells from human umbilical cord veins (Verweij, C. L. et al., Nucleic Acids Res. 13 (1985) 4699-4717), or from human platelet RNA, for example, according to the protocol described by Ware et al. (Proc. Natl. Acad. Sci. 88 (1991) 2946-2950). The cellular RNA is purified using the extraction technique with guanidine thiocyanate initially described by Cathala et al. (DNA 4 (1983) 329-335) and used as template for the synthesis of complementary DNA (cDNA) including the part of vWF to be amplified. In a first step, the synthesis of the noncoding strand is carried out using the kit distributed by Amersham and an oligodeoxynucleotide which is complementary to the nucleotide sequence of the mRNA coding for the contiguous residues located at the C-terminal of the part to be amplified. The resulting solution is then subjected to three enzymatic amplification cycles by the PCR technique using as primer the preceding oligodeoxynucleotide and a oligodeoxynucleotide identical to the nucleotide sequence coding for contiguous residues located at the N-terminal of the part of vWF to be amplified. The amplified fragments are then cloned in vectors of the M13 type for their verification by sequencing using either the universal primers located on both sides of the cloning multisite, or specific oligodeoxynucleotides of the amplified region of the vWF gene for which the sequence of several isomorphs is known (Sadler, J. E. et al., Proc. Natl. Acad. Sci. 82 (1985) 6394-6398; Verweij, C. L. et al., EMBO J. 5 (1986) 1839-1847; Shelton-Inloes, B. B. et al., Biochemistry 25 (1986) 3164-3171; Bonthron, D. et al., Nucleic Acids Res. 17 (1986) 7125-7127). Plasmid pET-8c52K is particularly useful because it comprises a fragment of the cDNA of vWF coding for residues 445-733 of human vWF and notably it includes the peptides G10 and D5, which are antagonists of the interaction between vWF and GP1b (Mori, H. et al., J. Biol. Chem. 263 (1988) 17901-17904). The fragment of vWF present in plasmid p5E is identical to the fragment of vWF of plasmid pET-8c52K except that the cysteine residues at positions 459, 462, 464, 471 and 474 were mutated into glycine residues by directed mutagenesis. Plasmid p7E is identical to plasmid p5E except that the cysteine

residues at positions 509 and 695 were also mutated into glycine residues by directed mutagenesis.

Example 2: Construction of a MstII-HindIII restriction fragment including a binding site of vWF to blood platelets

E.2.1 Peptide of the type P1-X-P2

E.2.1.1 Thr470-Val713 residues of vWF

The PCR amplification of the plasmid pET-5c52K with the oligodeoxynucleotides 5'-CCCGGGATCCCTTAGGCTTAACCTGTGAAGCCTGC-3' (Sq1969, the BamHI and MstII sites are underlined) and 5'-CCCGGGATCCAAGCTTAGACTTGTGCCATGTCG-3' (Sq2029, the BamHI and HindIII sites are underlined), generates a fragment which includes the Thr470 to Val713 residues of vWF (see Figure 1, Thr586 to Val829 residues). The amplified fragments are first cut with BamHI, cloned in the BamHI site of a vector of the pUC type, and the sequence of a clone is verified by sequencing. The peptide sequence so amplified comprises an MstII-HindIII restriction fragment including the Thr470 to Val713 residues of vWF, whose peptide sequence is identical to the corresponding sequence described by Titani et al. (Biochemistry 25 (1986) 3171-3184). Plasmid pYG1220 comprises this MstII-HindIII restriction fragment preceded by the HindIII-MstII fragment of plasmid pYG404 (see Example 4 and Figure 3B).

E.2.1.2 Residues Thr470-Pro704 of vWF

Residue 705 of natural vWF is O-glycosylated and located inside peptide D5, defined by the Leu694 to Pro708 residues of vWF (Mori, H. et al., J. Biol. Chem. 263 (1988) 17901-17904). In addition, it is known that a treatment of natural vWF with neuraminidase, whose function is to release the terminal sialic acids of the glycosylations of mammalian cells, allows the exposure of the binding sites of vWF to platelet GPIb in the absence of platelet agglutination factors such as botrocetin, for example. Thus, it is possible that the suppression of all or part of the O-glycosylation sites of recombinant vWF, and notably secreted by a yeast where it is assumed that the O-glycosylation lacks sialic acids, generates a product which is intrinsically capable of recognizing the platelet GPIb in the absence of such cofactors. A MstII-HindIII fragment including the Thr470 to Pro704 residues of vWF is thus generated in a manner similar to the preceding example: the fragments resulting from the PCR amplification of the plasmid p5E with the oligodeoxynucleotides 5'-CCCGGGATCCCTTAGGCTTAACCGGTGAAGCCGGC-3' (Sq2149, the BamHI and MstII sites are underlined) and 5'-CCATGGATCCAAGCTTAAGGA-GGAGGGGCTTCAGGGGCAAGGTC-3' (Sq2622, the BamHI and HindIII sites are underlined) are first cloned in a vector of the pUC type in the form of a BamHI restriction fragment, and the sequence of a clone is verified by sequencing. The sequence of the MstII-HindIII fragment so

generated corresponds to the corresponding sequence given in Figure 1 except that the codon TAA specifying the translation stop is located immediately downstream of the Pro704 residue of vWF and residues 471 and 474 are glycine residues and not cysteine residues. Plasmid pYG1310 comprises this MstII-HindIII restriction fragment preceded by the HindIII-MstII fragment of plasmid pYG404 (see Example 4 and Figure 3B).

E.2.1.3 Leu494-Pro704 residues of vWF

The peptide sequence present in plasmid pYG1310 also has threonine or serine residues in positions 485, 492, 493 and 500 which are naturally O-glycosylated in the native molecule of human vWF, located in the immediate proximity of peptide G10 defined by Mori et al. (J. Biol. Chem. 263 (1988) 17901-17904). The amplification by the PCR technique of the plasmid pET8C-52K by the oligodeoxynucleotides 5'-CCCGGGTACCTTAGGCTTACTGTATGTGGA-GGACATC-3' (Sq3037, the KpnI and MstII sites are underlined) and 5'-CCATGGATCCAAGC-TTAAGGAGGAGGGGCTTCAGGGGCAAGGTC-3' (Sq2622, the BamHI and HindIII sites are underlined) generates a fragment including the Leu494 to Pro704 residues of vWF. The amplified fragments are first cut by the KpnI and BamHI enzymes to be cloned in a vector of the pUC type cut by the same enzymes. A particular clone is isolated which corresponds to the expected sequence verified by sequencing. This KpnI-BamHI fragment thus comprises a MstII-HindIII fragment including the Leu494 to Pro704 residues of human vWF. Plasmid pYG1373 comprises this MstII-HindIII restriction fragment preceded by the HindIII-MstII fragment of plasmid pYG404 (see Example 4 and Figure 3B).

E.2.1.4 Tyr508-Pro704 residues of vWF

The peptide sequence present after PCR amplification in plasmid pYG1373 still has the threonine residue in position 500, which is naturally O-glycosylated in the native molecule of human vWF. The amplification by the PCR technique of plasmid pET8C-52K by the oligodeoxynucleotides 5'-CCCGGGTACCTTAGGCTTATACTGCAGCAGGCTACTGGACC-TG-3' (Sq2621, the KpnI and MstII sites are underlined) and 5'-CCATGGATCCAAGCTTAAG-GAGGAGGGGCTTCAGGGGCAAGGTC-3' (Sq2622, the BamHI and HindIII sites are underlined) generates a fragment including the Tyr508 to Pro704 residues of vWF. The amplified fragments are first cut by the KpnI and BamHI enzymes to be cloned in a vector of the pUC type cut by the same enzymes. A particular clone is isolated which corresponds to the expected sequence verified by sequencing. This KpnI-BamHI fragment thus comprises a MstII-HindIII fragment including the Tyr508 to Pro704 residues of human vWF. Plasmid pYG1309 comprises this MstII-HindIII restriction fragment preceded by the HindIII-MstII fragment of plasmid pYG404 (see Example 4 and Figure 3B).

E.2.1.5 Residues Pro502-Pro704 of vWF

The peptide sequence corresponding to the Pro502 to Pro704 residues of human vWF is generated from the preceding plasmid by the insertion of the oligodeoxynucleotides 5'-TTAGGGTTACCACCTTTGCATGACTTCTACTGCA-3' (Sq2751) and 5'-GTAGAAGTCATGCAAAGGTGGTAACCC-3' (Sq2752) which by pairing can be cloned between the MstII and PstI sites of the plasmid obtained after PCR amplification according to Example E.2.1.4, which allows the generation of a MstII-HindIII restriction fragment including the Pro502 to Pro704 residues of human vWF. Plasmid pYG1350 comprises this MstII-HindIII restriction fragment preceded by the HindIII-MstII fragment of plasmid pYG404 (see Example 4 and Figure 3B).

E.2.2 Thr470-Asp498 residues of vWF: peptide of type P1

In a particular embodiment, the binding site of vWF is a peptide including the Thr470 to Asp498 residues of mature vWF. This sequence, included in peptide G10 (Cys474-Pro488), was described by Mori et al. (J. Biol. Chem. 263 (1988) 17901-17904) and it is capable of antagonizing the interaction of human vWF with GP1b of human platelets. The sequence including peptide G10 is first generated in the form of a MstII-HindIII restriction fragment, for example by means of the PCR amplification technique or directly with the aid of synthetic oligodeoxynucleotides. For example, the PCR amplification products of plasmid pET-8c52K with the oligodeoxynucleotides Sq1969 and 5'-CCCGGGATCCAAGCTTAGTCCTCCACATA-CAG-3' (Sq1970, the BamHI and HindIII sites are underlined) are first cut by the BamHI enzyme and then cloned in the BamHI site of a vector of the pUC type. A particular clone is isolated which corresponds to the expected sequence verified by sequencing. This BamHI fragment thus comprises a MstII-HindIII fragment including the Thr470 to Asp498 residues of human vWF. Plasmid pYG1210 comprises this MstII-HindIII restriction fragment preceded by the HindIII-MstII fragment of plasmid pYG404 (see Example 4 and Figure 3B).

E.2.3 Residues Leu694-Pro708 of vWF: peptide of type P2

In a second embodiment, the binding site of vWF to GP1b is directly devised with the aid of synthetic oligodeoxynucleotides, and for example the oligodeoxynucleotides 5'-TTAGGCCTCTGTGACCTTGCCCCTGAAGCCCCTCCTCCTACTCTGCCCCCCTAAGC-TTA-3' and 5'-GATCTAAGCTTAGGGGGGCAGAGTAGGAGGAGGGGCTTCAGGGGCA-AGGTCACAGAGGCC-3'. These oligodeoxynucleotides form by pairing an MstII-BglII restriction fragment including the MstII-HindIII fragment corresponding to peptide D5 defined by the Leu694 to Pro708 residues of vWF (Mori, H. et al., J. Biol. Chem. 263 (1988) 17901-

17904). Plasmid pYG1204 comprises this MstII-HindIII restriction fragment preceded by the HindIII-MstII fragment of plasmid pYG404 (see Example 4 and Figure 3B).

E.2.4 Peptide of the type P1-XD-P2

Useful variants of plasmid pET-8c52K are deleted by directed mutagenesis between peptides G10 and D5, for example the collagen and/or heparin and/or botrocetin and/or sulfatide and/or ristocentin binding sites. An example is plasmid pMMB9, deleted by directed mutagenesis between the Cys509 and Ile662 residues. PCR amplification of this plasmid with the oligodeoxynucleotides Sq1969 and Sq2029 generates a MstII-HindIII restriction fragment including the Thr470 to Tyr508 and Arg663 to Val713 residues and in particular peptides G10 and D5 of vWF, and in particular its collagen binding site located between the Glu542 and Met622 residues is deleted (Roth, G. J. et al., Biochemistry 25 (1986) 8357-8361). Plasmid pYG1217 comprises this MstII-HindIII restriction fragment preceded by the HindIII-MstII fragment of plasmid pYG404 (see Example 4 and Figure 3B). In other embodiments, the use of combined techniques of directed mutagenesis and PCR amplification allows the generation at variants will of the MstII-HindIII restriction fragment of Figure 1, except that one or more sulfatide and/or botrecetine and/or heparin and/or collagen binding sites has (have) been deleted.

E.2.5 Peptide of type P1-X*-P2

E.2.5.1 Conformational alteration by substitution of the cysteine residues

The PCR amplification products of plasmids p5E and p7E with the oligodeoxynucleotides Sq2149 (5'-CCCGGGATCCCTTAGGCTTAACCGGTGAAGCCGGC-3', the BamHI and MstII sites are underlined) and Sq2029 are first cloned in a vector of the pUC type in the form of a BamHI restriction fragment, and the sequence of a clone is verified by sequencing. The sequence of the MstII-HindIII fragment so generated corresponds to the corresponding sequence given in Figure 1 except that residues 471 and 474 of vWF are glycine residues and not cysteine residues. Plasmid pYG1271 comprises this MstII-HindIII restriction fragment preceded by the HindIII-MstII fragment of plasmid pYG404 (see Example 4 and Figure 3B). Plasmid pYG1269 is generated in a similar manner except that plasmid p7E is used as the template in the PCR amplification by the oligodeoxynucleotides Sq2149 and Sq2029.

E.2.5.2 Conformational alteration by introduction of mutations of type IIB

Other particularly useful mutations concern at least one residue involved in pathologies of type IIB associated with vWF (increase in the intrinsic affinity of vWF for GP1b), such as the Arg543, Arg545, Trp550, Val551, Val553, Pro574 or Arg578 residues for example. The in vitro genetic recombination techniques also allow the introduction at will of one or more

supplementary residues in the sequence of vWF, and for example a supernumerary methionine between the positions Asp539 and Glu542. In a particular example, the mutations Arg543>Trp543 (R543W) and Pro574->Leu574 (P574L) are introduced by directed mutagenesis with the aid of the oligodeoxynucleotides 5'-GTGCTGAAGGCCTTTGTGGTCGACATGATG-GAGTGGCTGCGGATATCCCAGAAGTGGGTAGCGGTGGCCGTGGTGGAGTACC-3' (Sq2851; the codon specifying the Arg543 residue is underlined) and 5'-GGGCTCAAGGACC-GGAAGCGCTTAAGCGAGCTGCGGCGCATTGCCAGCCAG-3' (Sq2855; the codon specifying the residue Leu574 is underlined), respectively. After verification of the nucleotide sequence, one thus generates MstII-HindIII restriction fragments including the mutants of type IIB of human vWF R543W and P2574L. Plasmids pYG1359 (R543W) and pYG1360 (P574L) comprise these MstII-HindIII restriction fragments preceded by the HindIII-MstII fragment of plasmid pYG404 (see Example 4 and Figure 3B). The mutagenesis with the aid of the oligodeoxynucleotide Sq2851 also introduces the SalI, EcoRV and MluI sites at the Val538, Ile546 and Val551 positions, respectively. These restriction sites are not present in the corresponding natural sequence of human vWF and they are thus particularly useful to easily introduce any desirable mutation between the Val538 and Val551 residues. As an example, the oligodeoxynucleotides 5'-ATCCCAGAAGTGCGTA-3' (Sq3017, the codon specifying the mutant of type IIB Cys550 is underlined) and 5'-CGCGTACGCACTTCTGGGAT-3 (Sq3018) form by a pairing an EcoRV-MluI restriction fragment which can be cloned in plasmid pYG1359 cut by the EcoRV and MluI enzymes, which generate plasmid pYG1374 comprising the mutations R543W and W550C (Figure 3B). In the same manner, the oligodeoxynucleotides 5'-TCGACATGATGGAGCGGCTGCGGAT-3' (Sq3019, the codon specifying the Arg543 residue originating from the natural sequence is underlined) and 5'-ATCCGCAGCCGCTCCAT-CATG-3' (Sq3020) form by pairing an SalI-EcoRV restriction fragment which can be cloned in plasmid pYG1374 cut by the SalI and EcoRV enzymes, which generates plasmid pYG1386 which comprises only the mutation W550C (Figure 3B).

Example 3: Construction of a MstII/HindIII restriction fragment including a binding site of vWF to the subendothelium

In a particular embodiment, the binding sites of vWF to the components of the subendothelial tissue, and of collagen in particular, are generated by PCR amplification of plasmid pET-8c52K. For example, the use of the oligodeoxynucleotides Sq2258 (5'-GGATCC-TTAGGGCTGTGCAGCAGGCTACTGGACCTGGTC-3', the MstII site is underlined) and Sq2259 (5'-GAATTCAAGCTTAACAGAGGTAGCTAACGATCTCGTCCC-3', the HindIII site is underlined) allows the generation of plasmid pYG1254, whose MstII-HindIII restriction fragment includes the Cys509 to Cys695 residues of natural vWF (peptide of type X). The

ligation of this restriction fragment with the HindIII-MstII restriction fragment of plasmid pYG404 (see Example 4) generates the HindIII restriction fragment of plasmid pYG1276 (Figure 3B).

Molecular variants of types XD (see E.2.4) or X* (see E.2.5) can also be generated according to the same strategy, and they comprise any desirable combination between the binding sites of vWF to the sulfatides and/or to botrocetin and/or heparin and/or collagen and/or any residue responsible for a modification of the affinity of vWF for GP1b (pathologies of type II associated with vWF). In another embodiment, the domain capable of binding to collagen can also originate from the fragment of vWF between residues 911 and 1114 and described by Pareti et al. (J. Biol. Chem. (1987) 262: 13835-13841).

Example 4: Coupling at C-terminal of HSA

Plasmid pYG404 is described in the patent application EP 361 991. This plasmid comprises a HindIII restriction fragment coding for the prepro-HSA gene preceded by 21 nucleotides naturally present immediately upstream from the translation initiator ATG of the PGK gene of *S. cerevisiae*. This fragment comprises a HindIII-MstII restriction fragment corresponding to the totality of the gene coding for the HSA with the exception of the three amino acids closest to the C-terminal (leucine-glycine-leucine residues). The ligation of this fragment with any one of the MstII-HindIII fragments described in Examples 2 or 3 allows the generation of HindIII restriction fragments including composite genes coding for chimeric proteins in which a fragment of vWF having special properties is positioned in the translation reading frame at the C-terminal of the HSA molecule. Such composite genes are exemplified in the table of Figure 3B.

Example 5: Coupling at the N-terminal of HSA

In a particular embodiment, the combined techniques of directed mutagenesis and PCR amplification allow the construction of hybrid genes coding for a chimeric protein resulting from the translational coupling between a signal peptide (and, for example, the prepro region of HSA), a sequence including a fragment of vWF which has properties of adhesion and the mature form of the HSA or one of its molecular variants. These hybrid genes are preferably flanked at 5' of the translation initiation ATG and at 3' of the translation stop codon by HindIII restriction sites, which allows the generation of expression plasmids for these chimeric proteins, for example according to the strategy detailed in the following example.

Example 6: Expression plasmids

The chimeric proteins of the preceding examples can be expressed in yeasts from functional regulable or constitutive promoters such as for example those present in plasmids pYG105 (LAC4 promoter of *Kluyveromyces lactis*), pYG106 (PGK promoter of *Saccharomyces cerevisiae*), pYG536 (PHO5 promoter of *S. cerevisiae*), or hybrid promoters such as those described in the patent application EP 361 991. Plasmids pYG105 and pYG106 are particularly useful here, because they allow the expression of genes coded by the HindIII restriction fragments of Examples E.4 and E.5 from functional promoters in *K. lactis*, regulable (pYG105) or constitutive (pYG106). Plasmid pYG105 corresponds to plasmid pKan707 described in the patent application EP 361 991 in which the unique HindIII restriction site located in the gene of resistance to geneticin (G418) was destroyed by directed mutagenesis while preserving an unchanged protein (oligodeoxynucleotide 5'-GAAATGCATAAGCTCTTGCCATTCTCACCG-3'). The SalI-SacI fragment coding for the URA3 gene of the mutated plasmid was then replaced by a SalI-SacI restriction fragment comprising an expression cassette consisting of the LAC4 promoter of *K. lactis* (in the form of a SalI-HindIII fragment) and of the terminator of the PGK gene of *S. cerevisiae* (in the form of a HindIII-SacI fragment). Plasmid pYG105 is mitotically very stable in the *Kluyveromyces* yeasts and a restriction map of it is given in Figure 3. Plasmids pYG105 and pYG106 differ only in the nature of the transcription promoter encoded by the SalI-HindIII fragment. As an example, the cloning "in the productive orientation" (defined as the orientation which places the "prepro" region of the albumin proximally with respect to the transcription promoter) of HindIII restriction fragments of plasmids pYG1220, pYG1310, pYG1373, pYG1309, pYG1350, pYG1210, pYG1204, pYG1217, pYG1269, pYG1271, pYG1359, pYG1360, pYG1374, pYG1386 and pYG1276, in the HindIII site of plasmid pYG105 generates the expression plasmids pYG1248, pYG1313, pYG1375, pYG1311, pYG1355, pYG1214, pYG1206, pYG1223, pYG1279, pYG1283, pYG1361, pYG1365, pYG1377, pYG1389 and pYG1277, respectively.

Example 7: Transformation of yeasts

The transformation of yeasts belonging to the genus *Kluyveromyces*, and in particular the MW98-8C and CBS 293.91 strains of *K. lactis*, is carried out for example using the whole cell treatment technique employing lithium acetate (Ito, H. et al., J. Bacteriol. 153 (1983) 163-168), adapted as follows. The growth of the cells is carried out at 28°C in 50 mL of YPD medium with stirring and until the optical density at 600 nm (OD_{600}) is between 0.6 and 0.8; the cells are harvested by centrifugation at low speed, washed in a sterile solution of TE (10mM Tris-HCl, pH 7.4; 1 mM EDTA), resuspended in 3-4 mL of lithium acetate (0.1M in TE) to obtain a cell density of approximately 2 x 10^8 cells/mL, and then incubated at 30°C for 1 h under moderate

stirring. Aliquots of 0.1 mL of the resulting suspension of competent cells are incubated at 30°C for 1 h in the presence of DNA and at a final concentration of 35% polyethylene glycol (PEG_{4000}, Sigma). After a 5 min thermal shock at 42°C, the cells are washed 2 times, resuspended in 0.2 mL sterile water and incubated for 16 h at 28°C in 2 mL of YPD medium to allow the phenotypic expression of the G418 resistance gene, expressed under the control of the P_{kl} promoter (see EP 361 991); 200 µL of the cell suspension are then spread on selective YPD Petri dishes (G418, 200 µg/mL). The Petri dishes are then incubated at 28°C and the transformants appear after 2-3 days of cell growth.

Example 8: Secretion of the chimeras

After selection on a rich medium supplemented with G418, the recombinant clones are tested for their capacity to secrete the proteins chimeric between HSA and vWF. Some clones corresponding to the CBS 293.91 strain, transformed, for example, with plasmids pYG1214 (HSA-P1), pYG1206 (HSA-P2), pYG1223 (HSA-P1-XD-P2) and pYG1248 (HSA-P1-X-P2) or pKan707 (control vector) are incubated in YPD or YPL medium at 28°C. The cell supernatants are recovered by centrifugation when the cells reach the stationary growth phase, they are optionally concentrated 10 times by precipitation for 30 min at –20°C at a final concentration of 60% ethanol, and then tested after electrophoresis on an 8.5% SDS-PAGE gel or directly by staining the gel with Coomassie blue, or after immunoblot using, as primary antibodies, mouse antibodies directed against vWF or a polyclonal rabbit serum directed against HSA. In the immunological detection experiments, the nitrocellulose filter is first incubated in the presence of specific primary antibodies, washed several times, incubated in the presence of sheep antimouse antibodies (anti-vWF immunoblot) or sheep antirabbit antibodies (anti-HSA immunoblot), and then incubated in the presence of an avidinperoxidase complex using the "ABC kit" distributed by Vectastain (Biosys, S.A., Compiège, France). The immunological reaction is then developed by the addition of 3,3'-diaminobenzidine tetrahydrochloride (Prolabo) in the presence of hydrogen peroxide, according to the manufacturer's recommendations. The results of Figures 4-8 demonstrate that the yeast *K. lactis* is capable of secreting proteins chimeric between HSA and a fragment of vWF, and that these chimeras are recognized by antibodies specific for HSA or vWF.

Example 9: Purification and molecular characterization of the secreted products

The chimeras present in the culture supernatants correspond to the CBS 293.91 strain, transformed, for example, by the expression plasmids according to Example 6, are characterized in a first step by means of specific antibodies for the HSA part and the vWF part. The results of Figures 4-8 demonstrate that the yeast *K. lactis* is capable of secreting proteins chimeric between

HSA and the fragment of vWF, and that these chimeras are immunologically reactive. It can also be desirable to purify some of these chimeras. The culture is then centrifuged (10,000 G, 30 min), the supernatant is passed through a 0.22 mm [sic; μm] filter (Millipore), and then concentrated by ultrafiltration (Amicon) using a membrane whose discrimination threshold is at 30 kd. The concentrate obtained is then dialized against a solution of Tris-HCl (50 mM, pH 8) and then purified on a column. For example, the concentrate corresponding to the culture supernatant of the CBS 293.91 strain transformed by plasmid pYG1206 is purified by affinity chromatography on Blue-Trisacryl (IBF). Purification by ion-exchange chromatography can also be used. For example, in the case of the chimera HSA-vWF470-713, the concentrate obtained after ultrafiltration is dialyzed against a solution of Tris-HCl (50 mM, pH 8) and then deposited in 20 mL fractions on a column (5 mL) of a cation-exchanger (S Fast Flow, Pharmacia) equilibrated in the same buffer. The column is then washed several times with the solution of Tris-HCl (50 mM, pH 8) and the chimeric protein is then eluted from the column by a gradient (0-1M) of NaCl. The fractions containing the chimeric protein are then combined and dialyzed against a solution of Tris-HCl 50 mM (pH 8), and then reapplied to an S Fast Flow column. After elution of the column, the fractions containing the protein are combined, dialyzed against water, and lyophilized before characterization: for example, the sequencing (Applied Biosystem) of the protein (HSA-vWF470-704 C471G, C474G) secreted by the yeast CBS 293.91 gives the expected N-terminal sequence of HSA (Asp-Ala-His...), demonstrating a correct maturation of the chimera immediately at the C-terminal of the doublet of the Arg-Arg residues of the "pro" region of HSA (Figure 1). The essentially monomeric character of the chimeric proteins between HSA and vWF is also confirmed by their elution profile on a TSK 3000 column (Toyo Soda Company, equilibrated by a solution of cacodylate (pH 7) containing 0.2M Na_2SO_4): for example, the chimera (HSA-vWF 470-704 C471G, C474G) behaves under these conditions like a protein having an apparent molecular weight of 95 kd, demonstrating its monomeric character.

Example 10: Antagonist activity of the genetic hybrids between HSA and vWF for platelet agglutination

The antagonistic activity of the products is determined by measuring the dose-dependent inhibition of the agglutination of human platelets fixed by paraformaldehyde according to the method described by Prior et al. (Bio/Technology (1992) 10: 66). The measurements are carried out in aggregometer (PAP-4, Bio Data, Horsham, PA, USA), which records the variations over time in the optical transmission under stirring at 37°C in the presence of vWF, botrocetin (8.2 mg/mL) and of the product to be tested at different dilutions (concentrations). For each measurement, 400 mL (8×10^7 platelets) of a suspension of human platelets stabilized with paraformaldehyde (0.5%, then resuspended in (NaCl (137 mM); $MgCl_2$ (1 mM); NaH_2PO_4

(0.36 mM); $NaHCO_3$ (10 mM); KCl (2.7 mM); glucose (5.6 mM); HSA (3.5 mg/mL); HEPES buffer (10 mM, pH 7.35)) are preincubated at 37°C in the cylindrical cuvette (8.75 x 50 mm, Wellcome Distriwell, 159 rue Nationale, Paris) of the aggregometer for 4 min, and then 30 mL of the solution of the product to be tested are added at different dilutions in an apyrogenic formulation vehicle (mannitol (50 g/L); citric acid (192 mg/L); L-lysine monohydrochloride (182.6 mg/L); NaCl (88 mg/L); pH adjusted to 3.5 by the addition of NaOH (1M)), or of formulation vehicle only (control test). The resulting suspension is then incubated for 1 min at 37°C and one adds 12.5 mL of human vWF (American Bioproducts, Parsippany, NJ, USA; 11% of von Willebrand activity measured according to the recommendations for use of the PAP-4 (Platelet Aggregation Profiler®) with the aid of platelets fixed in formaldehyde (2×10^5 platelets/mL), human plasma containing 0-100% vWF and ristocentin (10 mg/mL, see pp. 36-45: vW Program™) which one incubates at 37°C for 1 min before adding 12.5 mL of the botrocetin solution (purified from lyophilized venom from *Bothrops jararaca* (Sigma), according to the protocol described by Sugimoto et al., Biochemistry (1991) 266; 18172). The recording of the reading of the transmission as a function of time is then carried out for 2 min under stirring with the aid of a magnetized bar (Wellcome Distriwell) placed in the cuvette and under magnetic stirring at 1100 rpm ensured by the aggregometer. The mean variation in the optical transmission ($n^3 5$ for each dilution) over time is thus a measurement of the platelet agglutination due to the presence of vWF and of botrecetine, in the absence or in the presence of variable concentrations of the product to be tested. From such recordings one then determines the % inhibition of the platelet agglutination due to each concentration of the product, and one traces the straight line giving the % inhibition as a function of the inverse of the dilution of the product on a log-log scale. The IC_{50} (or concentration of product causing 50% inhibition of the agglutination) is then determined on this straight line. The table of Figure 9 compares the IC_{50} of several HSA-vWF chimeras of the present invention and it demonstrates that some of them are better antagonists of platelet agglutination than the product RG12986 described by Prior et al. (Bio/Technology (1992) 10: 66) and included in the tests as a calibration value. Identical tests of the inhibition of the agglutination of human platelets in the presence of vWF from porcine plasma (Sigma) in addition allows the demonstration that some of the hybrids of the present invention, and notably some variants of type IIB, are very good antagonists of platelet agglutination in the absence of cofactors of the botrocetin type. The botrocetin-independent antagonism of these particular chimeras can also be demonstrated according to the protocol initially described by Ware et al. (Proc. Natl. Acad. Sci. (1991) 88: 2946) by displacement of the monoclonal antibody ^{125}I-LJ-IB1 (10 mg/mL), a competitive inhibitor of the binding of vWF to platelet GPIb (Handa, M. et al., (1986) J. Biol. Chem. 261: 12579) after 30 min of incubation at 22°C in the presence of fresh platelets (10^8 platelets/mL).

List of sequences

(2) Information for SEQ ID NO: 1:

(i) Characteristics of the sequence:

(A) Length: 2591 bp

(B) Type: nucleic acid

(C): Number of strands: double

(D): Configuration: linear

(ii) Type of molecule: cDNA

(iii) Hypothetical: no

(iii) Antisense: no

(ix) Additional characteristic:

(A) Name/key: CDS

(B) Location: 26..2587

Claims

1. Recombinant polypeptide consisting of an adhesive part derived from the structure of vWF capable of at least partially antagonizing the binding of vWF to platelets and/or the subendothelium, and of a part allowing its stabilization and in vivo presentation.

2. Polypeptide according to Claim 1, characterized in that the adhesive part consists in its entirety or in part of the peptide sequence between residues 445 and 733 of vWF or a variant thereof.

3. Polypeptide according to Claim 2, characterized in that the adhesive part presents a structure chosen from:

(a) the peptide sequence between residues 445-733 of vWF, or,

(b) a part of peptide sequence (a) capable of at least partially antagonizing the binding of vWF to GP1b and/or to the subendothelium, or

(c) a structure derived from structures (a) or (b) by structural modifications (mutation, substitution, addition and/or deletion of one or more residues) and capable of at least partially antagonizing the binding of vWF to GP1b and/or to the subendothelium, or

(d) a non-natural peptide sequence, for example one isolated from random peptide banks, and capable of at least partially antagonizing the binding of vWF to GP1b and/or the subendothelium.

4. Polypeptide according to Claim 3, characterized in that the adhesive part consists of a sequence chosen from the peptides of type P1, P2, X, XD and X* or any combination of these peptides with each other.

5. Polypeptide according to Claim 4, characterized in that the combination of peptides is chosen from the peptides of type P1-P2, P1-X, P1-XD, P1-X*, X-P2, XD-P2, X*-P2, P1-X-P2, P1-XD-P2 and P1-X*-P2.

6. Polypeptide according to Claim 4, characterized in that the adhesive part consists of any peptide of a type defined in Claims 4 and 5 represented more than once.

7. Polypeptide according to one of Claims 1-6, characterized in that the adhesive part is coupled to the N-terminal end of the stabilizing structure.

8. Polypeptide according to one of Claims 1-6, characterized in that the adhesive part is coupled to the C-terminal end of the stabilizing structure.

9. Polypeptide according to one of Claims 1-8, characterized in that the stabilizing structure is a polypeptide having a high plasma half-life.

10. Polypeptide according to Claim 9, characterized in that the polypeptide having a high plasma half-life is a protein such as albumin, apolipoprotein, an immunoglobulin or a transferrin.

11. Polypeptide according to Claim 9, characterized in that the polypeptide having a high plasma half-life is derived by structural modification(s) (mutation, substitution, addition and/or deletion of one or more residues, chemical modification) of a protein according to Claim 10.

12. Polypeptide according to one of Claims 9-11, characterized in that the stabilizing structure is a weakly immunogenic or nonimmunogenic polypeptide for the organism in which it is used.

13. Polypeptide according to Claim 9, characterized in that the stabilizing structure is an albumin or a variant of albumin.

14. Nucleotide sequence coding for a polypeptide according to any one of Claims 1-13.

15. Nucleotide sequence according to Claim 14, characterized in that it comprises a "leader" sequence allowing the secretion of the polypeptide expressed.

16. Expression cassette comprising a nucleotide sequence according to one of Claims 14 or 15 under the control of a transcription initiation region and optionally a transcription termination region.

17. Self-replicating plasmid comprising an expression cassette according to Claim 16.

18. Eukaryotic or prokaryotic recombinant cell into which a nucleotide sequence according to one of Claims 14 or 15, or an expression cassette according to Claim 16, or a plasmid according to Claim 17, has been inserted.

19. Recombinant cell according to Claim 18, characterized in that it is a yeast, an animal cell, a fungus or a bacterium.

SEQ ID NO: 1

```
AAGCT TTACAACAAA TATAAAAACA ATG AAG TGG GTA ACC TTT ATT TCC CTT CTT TTT CTC TTT
                            Met Lys Trp Val Thr Phe Ile Ser Leu Leu Phe Leu Phe   -12

AGC TCG GCT TAT TCC AGG GGT GTG TTT CGT CGA GAT GCA CAC AAG AGT GAG GTT GCT CAT
Ser Ser Ala Tyr Ser Arg Gly Val Phe Arg Arg Asp Ala His Lys Ser Glu Val Ala His     9

CGG TTT AAA GAT TTG GGA GAA GAA AAT TTC AAA GCC TTG GTG TTG ATT GCC TTT GCT CAG
Arg Phe Lys Asp Leu Gly Glu Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe Ala Gln    29

TAT CTT CAG CAG TGT CCA TTT GAA GAT CAT GTA AAA TTA GTG AAT GAA GTA ACT GAA TTT
Tyr Leu Gln Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn Glu Val Thr Glu Phe    49

GCA AAA ACA TGT GTT GCT GAT GAG TCA GCT GAA AAT TGT GAC AAA TCA CTT CAT ACC CTT
Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu Asn Cys Asp Lys Ser Leu His Thr Leu    69

TTT GGA GAC AAA TTA TGC ACA GTT GCA ACT CTT CGT GAA ACC TAT GGT GAA ATG GCT GAC
Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu Arg Glu Thr Tyr Gly Glu Met Ala Asp    89

TGC TGT GCA AAA CAA GAA CCT GAG AGA AAT GAA TGC TTC TTG CAA CAC AAA GAT GAC AAC
Cys Cys Ala Lys Gln Glu Pro Glu Arg Asn Glu Cys Phe Leu Gln His Lys Asp Asp Asn   109

CCA AAC CTC CCC CGA TTG GTG AGA CCA GAG GTT GAT GTG ATG TGC ACT GCT TTT CAT GAC
Pro Asn Leu Pro Arg Leu Val Arg Pro Glu Val Asp Val Met Cys Thr Ala Phe His Asp   129

AAT GAA GAG ACA TTT TTG AAA AAA TAC TTA TAT GAA ATT GCC AGA AGA CAT CCT TAC TTT
Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg Arg His Pro Tyr Phe   149

TAT GCC CCG GAA CTC CTT TTC TTT GCT AAA AGG TAT AAA GCT GCT TTT ACA GAA TGT TGC
Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg Tyr Lys Ala Ala Phe Thr Glu Cys Cys   169

CAA GCT GCT GAT AAA GCT GCC TGC CTG TTG CCA AAG CTC GAT GAA CTT CGG GAT GAA GGG
Gln Ala Ala Asp Lys Ala Ala Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly   189

AAG GCT TCG TCT GCC AAA CAG AGA CTC AAG TGT GCC AGT CTC CAA AAA TTT GGA GAA AGA
Lys Ala Ser Ser Ala Lys Gln Arg Leu Lys Cys Ala Ser Leu Gln Lys Phe Gly Glu Arg   209

GCT TTC AAA GCA TGG GCA GTA GCT CGC CTG AGC CAG AGA TTT CCC AAA GCT GAG TTT GCA
Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Arg Phe Pro Lys Ala Glu Phe Ala   229

GAA GTT TCC AAG TTA GTG ACA GAT CTT ACC AAA GTC CAC ACG GAA TGC TGC CAT GGA GAT
Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys Val His Thr Glu Cys Cys His Gly Asp   249
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Figure 1 (a)

```
CTG CTT GAA TGT GCT GAT GAC AGG GCG GAC CTT GCC AAG TAT ATC TGT GAA AAT CAA GAT
Leu Leu Glu Cys Ala Asp Asp Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp   269

TCG ATC TCC AGT AAA CTG AAG GAA TGC TGT GAA AAA CCT CTG TTG GAA AAA TCC CAC TGC
Ser Ile Ser Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His Cys   289

ATT GCC GAA GTG GAA AAT GAT GAG ATG CCT GCT GAC TTG CCT TCA TTA GCT GCT GAT TTT
Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser Leu Ala Ala Asp Phe   309

GTT GAA AGT AAG GAT GTT TGC AAA AAC TAT GCT GAG GCA AAG GAT GTC TTC CTG GGC ATG
Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala Glu Ala Lys Asp Val Phe Leu Gly Met   329

TTT TTG TAT GAA TAT GCA AGA AGG CAT CCT GAT TAC TCT GTC GTA CTG CTG CTG AGA CTT
Phe Leu Tyr Glu Tyr Ala Arg Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg Leu   349

GCC AAG ACA TAT GAA ACC ACT CTA GAG AAG TGC TGT GCC GCT GCA GAT CCT CAT GAA TGC
Ala Lys Thr Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro His Glu Cys   369

TAT GCC AAA GTG TTC GAT GAA TTT AAA CCT CTT GTG GAA GAG CCT CAG AAT TTA ATC AAA
Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val Glu Glu Pro Gln Asn Leu Ile Lys   389

CAA AAT TGT GAG CTT TTT GAG CAG CTT GGA GAG TAC AAA TTC CAG AAT GCG CTA TTA GTT
Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu Tyr Lys Phe Gln Asn Ala Leu Leu Val   409

CGT TAC ACC AAG AAA GTA CCC CAA GTG TCA ACT CCA ACT CTT GTA GAG GTC TCA AGA AAC
Arg Tyr Thr Lys Lys Val Pro Gln Val Ser Thr Pro Thr Leu Val Glu Val Ser Arg Asn   429

CTA GGA AAA GTG GGC AGC AAA TGT TGT AAA CAT CCT GAA GCA AAA AGA ATG CCC TGT GCA
Leu Gly Lys Val Gly Ser Lys Cys Cys Lys His Pro Glu Ala Lys Arg Met Pro Cys Ala   449

GAA GAC TAT CTA TCC GTG GTC CTG AAC CAG TTA TGT GTG TTG CAT GAG AAA ACG CCA GTA
Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu Cys Val Leu His Glu Lys Thr Pro Val   469

AGT GAC AGA GTC ACC AAA TGC TGC ACA GAA TCC TTG GTG AAC AGG CGA CCA TGC TTT TCA
Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser Leu Val Asn Arg Arg Pro Cys Phe Ser   489

GCT CTG GAA GTC GAT GAA ACA TAC GTT CCC AAA GAG TTT AAT GCT GAA ACA TTC ACC TTC
Ala Leu Glu Val Asp Glu Thr Tyr Val Pro Lys Glu Phe Asn Ala Glu Thr Phe Thr Phe   509

CAT GCA GAT ATA TGC ACA CTT TCT GAG AAG GAG AGA CAA ATC AAG AAA CAA ACT GCA CTT
His Ala Asp Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala Leu   529

GTT GAG CTT GTG AAA CAC AAG CCC AAG GCA ACA AAA GAG CAA CTG AAA GCT GTT ATG GAT
Val Glu Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu Lys Ala Val Met Asp   549

GAT TTC GCA GCT TTT GTA GAG AAG TGC TGC AAG GCT GAC GAT AAG GAG ACC TGC TTT GCC
Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys Ala Asp Asp Lys Glu Thr Cys Phe Ala   569

                                                    MstII
GAG GAG GGT AAA AAA CTT GTT GCT GCA AGT CAA GCT GCC TTA GGC TTA ACC TGT GAA GCC
Glu Glu Gly Lys Lys Leu Val Ala Ala Ser Gln Ala Ala Leu Gly Leu Thr Cys Glu Ala   589
                                                    SAH<---I--->vWF
```

Figure 1 (b)

```
TGC CAG GAG CCG GGA GGC CTG GTG GTG CCT CCC ACA GAT GCC CCG GTG AGC CCC ACC ACT
Cys Gln Glu Pro Gly Gly Leu Val Val Pro Pro Thr Asp Ala Pro Val Ser Pro Thr Thr   609

                                                        PstI
CTG TAT GTG GAG GAC ATC TCG GAA CCG CCG TTG CAC GAT TTC TAC TGC AGC AGG CTA CTG
Leu Tyr Val Glu Asp Ile Ser Glu Pro Pro Leu His Asp Phe Tyr Cys Ser Arg Leu Leu   629

GAC CTG GTC TTC CTG CTG GAT GGC TCC TCC AGG CTG TCC GAG GCT GAG TTT GAA GTG CTG
Asp Leu Val Phe Leu Leu Asp Gly Ser Ser Arg Leu Ser Glu Ala Glu Phe Glu Val Leu   649

AAG GCC TTT GTG GTG GAC ATG ATG GAG CGG CTG CGC ATC TCC CAG AAG TGG GTC CGC GTG
Lys Ala Phe Val Val Asp Met Met Glu Arg Leu Arg Ile Ser Gln Lys Trp Val Arg Val   669

GCC GTG GTG GAG TAC CAC GAC GGC TCC CAC GCC TAC ATC GGG CTC AAG GAC CGG AAG CGA
Ala Val Val Glu Tyr His Asp Gly Ser His Ala Tyr Ile Gly Leu Lys Asp Arg Lys Arg   689

CCG TCA GAG CTG CGG CGC ATT GCC AGC CAG GTG AAG TAT GCG GGC AGC CAG GTG GCC TCC
Pro Ser Glu Leu Arg Arg Ile Ala Ser Gln Val Lys Tyr Ala Gly Ser Gln Val Ala Ser   709

ACC AGC GAG GTC TTG AAA TAC ACA CTG TTC CAA ATC TTC AGC AAG ATC GAC CGC CCT GAA
Thr Ser Glu Val Leu Lys Tyr Thr Leu Phe Gln Ile Phe Ser Lys Ile Asp Arg Pro Glu   729

GCC TCC CGC ATC GCC CTG CTC CTG ATG GCC AGC CAG GAG CCC CAA CGG ATG TCC CGG AAC
Ala Ser Arg Ile Ala Leu Leu Leu Met Ala Ser Gln Glu Pro Gln Arg Met Ser Arg Asn   749

TTT GTC CGC TAC GTC CAG GGC CTG AAG AAG AAG AAG GTC ATT GTG ATC CCG GTG GGC ATT
Phe Val Arg Tyr Val Gln Gly Leu Lys Lys Lys Lys Val Ile Val Ile Pro Val Gly Ile   769

GGG CCC CAT GCC AAC CTC AAG CAG ATC CGC CTC ATC GAG AAG CAG GCC CCT GAG AAC AAG
Gly Pro His Ala Asn Leu Lys Gln Ile Arg Leu Ile Glu Lys Gln Ala Pro Glu Asn Lys   789

GCC TTC GTG CTG AGC AGT GTG GAT GAG CTG GAG CAG CAA AGG GAC GAG ATC GTT AGC TAC
Ala Phe Val Leu Ser Ser Val Asp Glu Leu Glu Gln Gln Arg Asp Glu Ile Val Ser Tyr   809

CTC TGT GAC CTT GCC CCT GAA GCC CCT CCT CCT ACT CTG CCC CCC GAC ATG GCA CAA GTC
Leu Cys Asp Leu Ala Pro Glu Ala Pro Pro Pro Thr Leu Pro Pro Asp Met Ala Gln Val   829

TAA GCTT
***
```

Figure 1 (c)

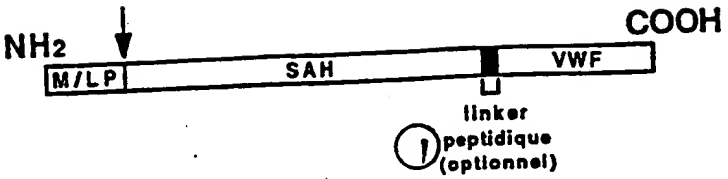


Figure 2B

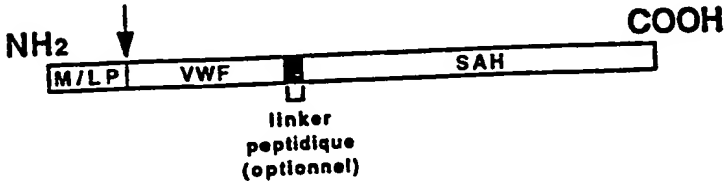


Figure 2C

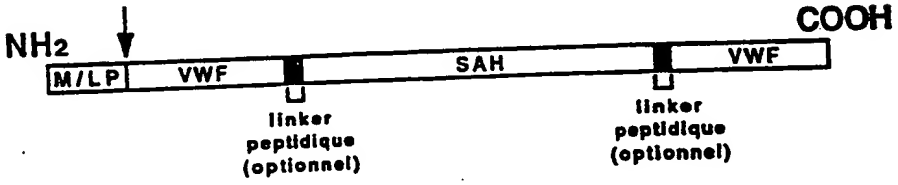


Figure 2

Key: 1 (optional) peptide linker

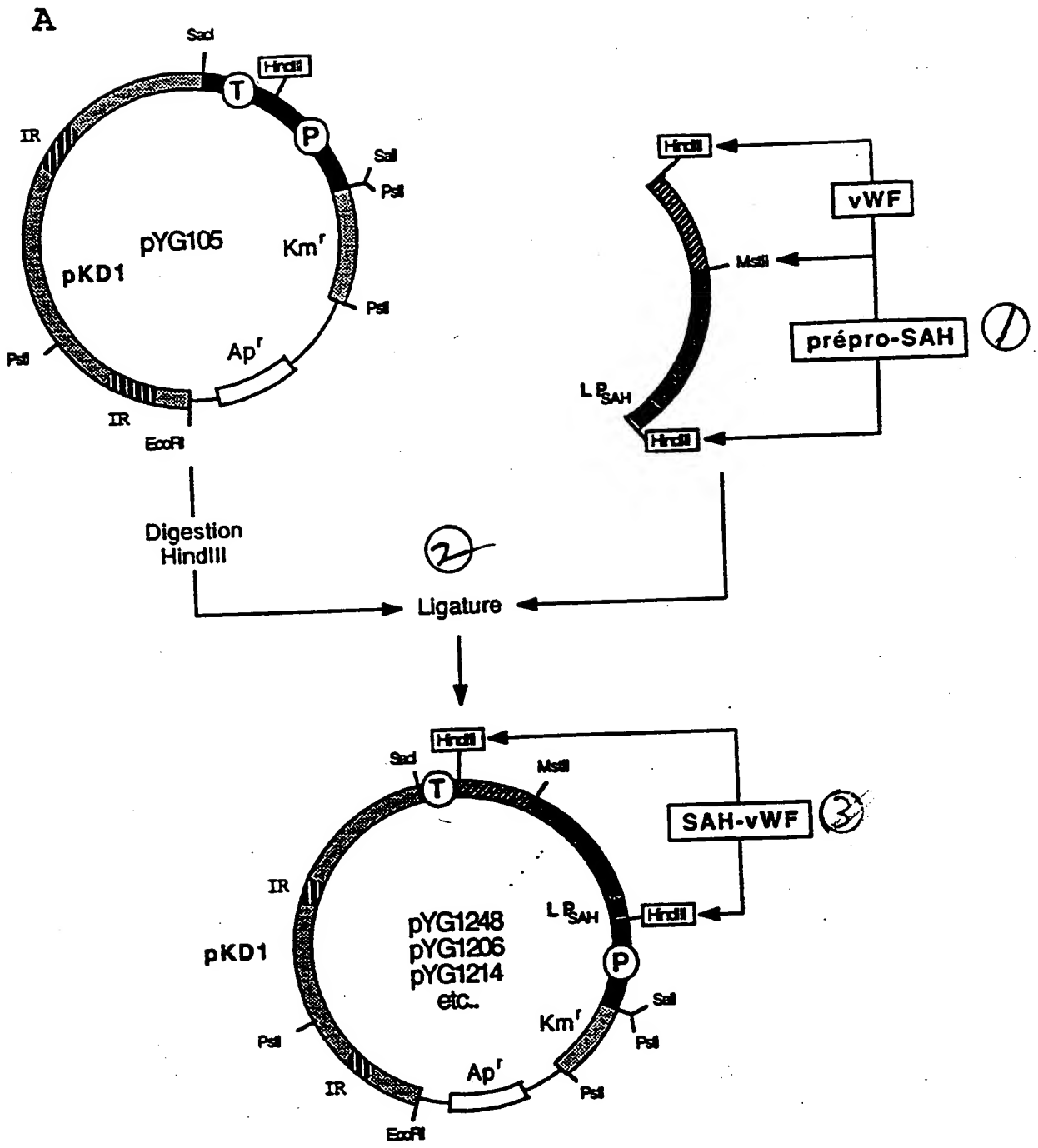


Figure 3 (a)

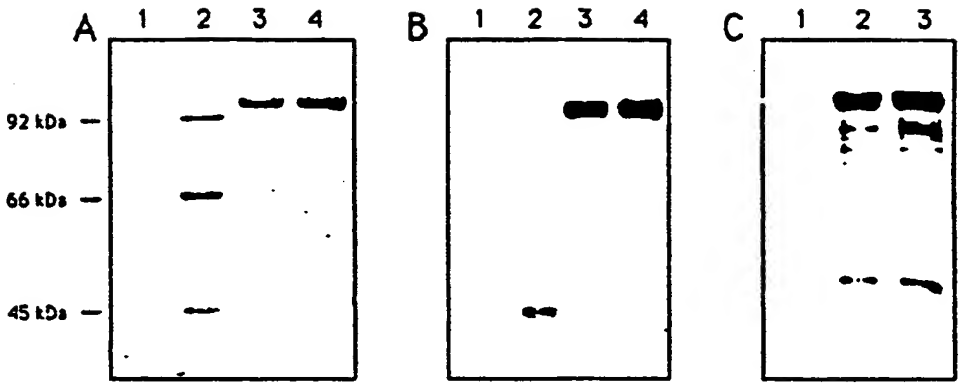
Key: 1 prepro HSA
2 Ligation
3 HAS-vWF

B

(1) PLASMIDE HSA-vWF	(2) STRATEGIE D'OBTENTION	(3) CARACTERISTIQUES	(4) O-GLYCOSYLATION POTENTIELLE	(5) PLASMIDE D'EXPRESSION
pYG1220	(6) PCR sur pET-8c52K (Sq1969+Sq2029)	vWF470->713	T485; T492; T493; S500; T705	pYG1248
pYG1310	(6) PCR sur p5E (Sq2149+Sq2622)	vWF470->704 C471G; C474G	T485; T492; T493; S500	pYG1313
pYG1373	(6) PCR sur pET-8c52K (Sq3037+Sq2622)	vWF494->704	S500	pYG1375
pYG1309	(6) PCR sur pET-8c52K (Sq2621+Sq2622)	vWF508->704	NONE	pYG1311
pYG1350	(7) substitution du fragment MstII-PstI de pYG1309 par Sq2751+Sq2752	vWF502->704	NONE	pYG1355
pYG1210	PCR sur pET-8c52K (Sq1969+Sq1970)	vWF470->498	T485; T492; T493;	pYG1214
pYG1204	(8) insertion du fragment MstII-BglII au site MstII de la SAH	vWF694->708	T705	pYG1206
pYG1217	PCR sur pMMB9 (Sq1969+Sq2029)	vWF470->713 Δ509-662	T485; T492; T493; S500; T705	pYG1223
pYG1269	PCR sur p7E (Sq2149+Sq2029)	vWF470->713 C471G; C474G; C509G; C695G	T485; T492; T493; S500; T705	pYG1279
pYG1271	PCR sur p5E (Sq2149+Sq2029)	vWF470->713 C471G; C474G	T485; T492; T493; S500; T705	pYG1283
pYG1359	(9) mutagénèse par Sq2851	vWF470->704 C471G; C474G; R543W	T485; T492; T493; S500	pYG1361
pYG1360	(9) mutagénèse par Sq2855	vWF470->704 C471G; C474G; P574L	T485; T492; T493; S500	pYG1365
pYG1374	(10) substitution du fragment EcoRV-MluI de pYG1359 par Sq3017+Sq3018	vWF470->704 C471G; C474G; R543W; W550C	T485; T492; T493; S500	pYG1377
pYG1386	(11) substitution du fragment SalI-EcoRV de pYG1374 par Sq3019+Sq3020	vWF470->704 C471G; C474G; W550C	T485; T492; T493; S500	pYG1389
pYG1276	(6) PCR sur pET-8c52K (Sq2258+Sq2259)	vWF509->695	NONE	pYG1277

Figure 3 (b)

Key:
1 HAS-vWF Plasmid
2 Strategy for obtaining
3 Characteristics
4 Potential O-glycosylation
5 Expression plasmid
6 On
7 Substitution of the MstII-PstI fragment of pYG1309 by Sq2751 + Sq2752
8 Insertion of the MstII-BglII fragment at the MstII site of HSA
9 Mutagenesis by
10 Substitution of the EcoRV-MluI fragment of pYG1359 by Sq3017 + Sq3018
11 Substitution of the SalI-EcoRV fragment of pYG1374 by Sq3019 + Sq3020



Figüre 4

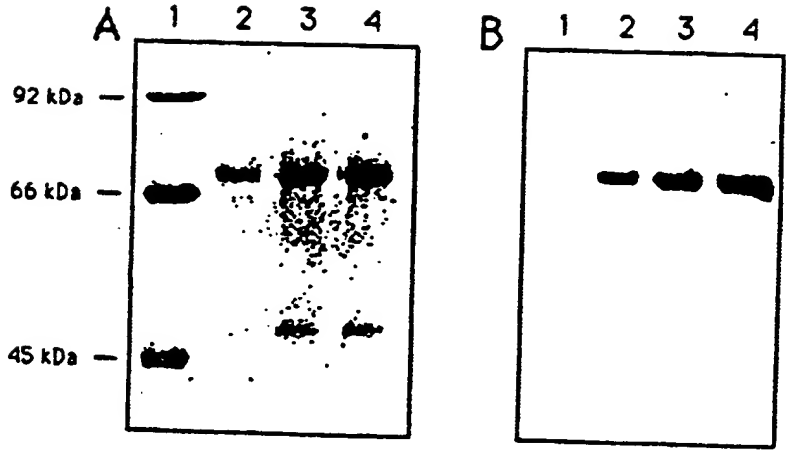


Figure 5

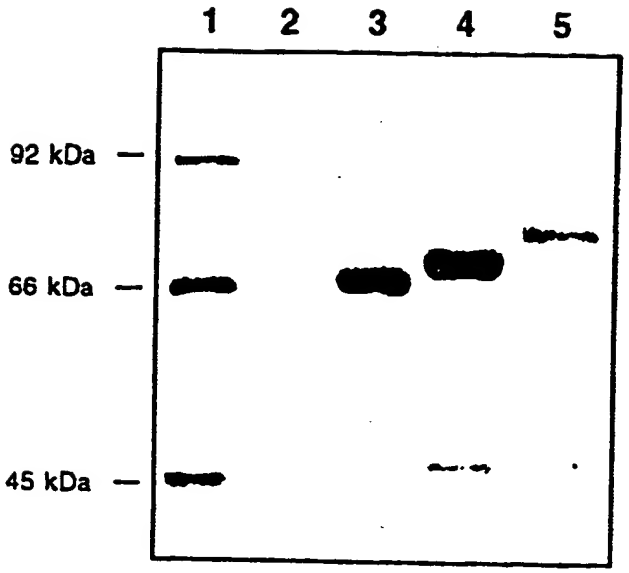


Figure 6

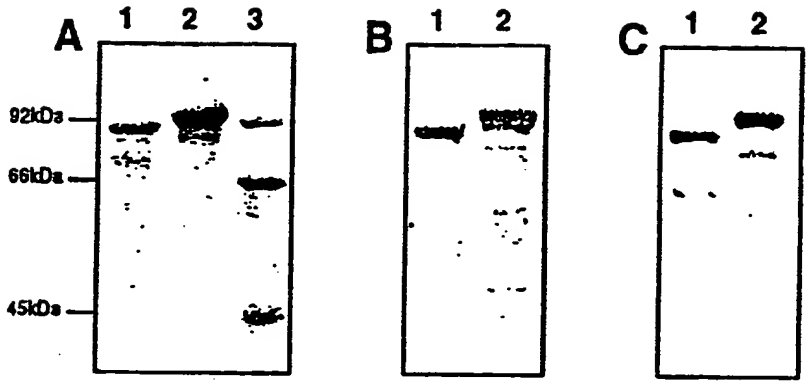


Figure 7

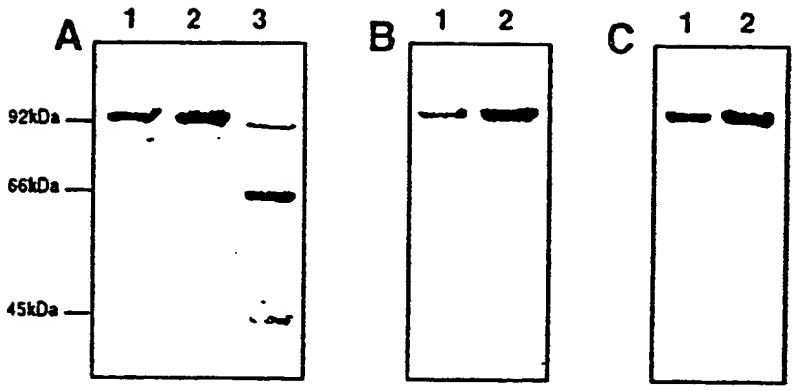


Figure 8

(1) PRODUIT	(2) IC_{50} (nM)
RG12986	50
(3) SAH-vWF$_{694-708}$	50000
(3) SAH-vWF$^{470-713}_{C471,474->G}$	20
(3) SAH-vWF$^{470-704}_{C471,474->G}$	<10

Key: 1 Product
2 IC_{50} (NM)
3 HSA

Figure 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FR93/00087

A. CLASSIFICATION OF SUBJECT MATTER

Int. cl.^{5} : C12N 15/12; C12N 15/62; C12N 15/14; C12N 5/10 A61K 37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl.^{5} : C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP, A, 0 255 206 (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 3 February 1988, cited in the application see the whole document	1-23
Y	WO, A, 9 113 093 (BIO-TECHNOLOGY GENERAL CORPORATION) 5 September 1991, see the whole document	1-23
Y	EP, A, 0 413 62 (RHONO-POULENC SANTE) 20 February 1991, cited in the application see the whole document	1-23
P,Y	WO, A, 9 300 357 (RHONE POULENC RORER INT HOLDING) 7 January 1993, see the whole document	1-23
P,Y	WO, A, 9 217 192 (THE SCRIPPS RESEARCH INSTITUTE) 15 October 1992, see the whole document	1-23
P,Y	WO, A, 9 206 999 (THE SCRIPPS RESEARCH INSTITUTE) 30 April 1992, see the whole document	1-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 June 1993 (16.06.93)	2 July 1993 (02.07.93)
Name and mailing address of the ISA/ European Patent Office Facsimile No.	**Authorized officer** Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FR93/00087

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF BIOLOGICAL CHEMISTRY . (MICROFILMS) Vol. 263, NO.34, 5 December 1988, BALTIMORE, MD US pages 17901-17904 MOHRI, H. ET AL. 'Structure of the von Willebrand Factor domain interacting with glycoprotein Ib' cited in the application see the whole document	1-23
Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. Vol. 164, No.3, 15 November 1989, DULUTH, MINNESOTA US pages 1339-1347 PIETU, G. ET AL. 'Production in Escherichia coli of a biologically active subfragment of von Willebrand Factor corresponding to the platelet glycoprotein Ib, collagen and heparin binding domains' see the whole document	1-23

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

FR 9300087
SA 70241

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0255206	03-02-88	AU-B-	617981	12-12-91
		AU-A-	7371587	03-12-87
		JP-A-	63152396	24-06-88
WO-A-9113093	05-09-91	AU-A-	7496491	18-09-91
		EP-A-	0517826	16-12-92
EP-A-0413622	20-02-91	FR-A-	2650598	08-02-91
		CA-A-	2022539	04-02-91
		JP-A-	3178998	02-08-91
WO-A-9300357	07-01-93	AU-A-	2297792	25-01-93
WO-A-9217192	15-10-92	AU-A-	1757592	02-11-92
		AU-A-	9069591	20-05-92
		WO-A-	9206999	30-04-92
WO-A-9206999	30-04-92	AU-A-	9069591	20-05-92
		AU-A-	1757592	02-11-92
		WO-A-	9217192	15-10-92

EPO FORM P0479